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United States Patent Application

of

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For

Pfu Replication Accessory Factors and Methods of Use

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Related Application Information

This application claims priority from U.S. Provisional Application Serial No. 60/146,580, filed July 30, 1999.

Background And Summary Of The Invention

The invention relates to the field of replicating, amplifying, and sequencing nucleic acids. Further, this invention relates to novel proteins that enhance the activity of the polymerases.

In vitro polymerization techniques have enormously benefited the fields of biotechnology and medicine. The ability to manipulate nucleic acids with polymerization reactions greatly facilitates techniques ranging from gene characterization and molecular cloning (including, but not limited to sequencing, mutagenesis, synthesis and amplification of DNA), determining allelic variations, and detecting and screening of various diseases and conditions (e.g., hepatitis B).

An *in vitro* polymerization technique of great interest is the polymerase chain reaction (PCR). This method rapidly and exponentially replicates and amplifies nucleic acids of interest. PCR is performed by repeated cycles of denaturing a DNA template, usually by high temperatures, then annealing opposing primers to the complementary DNA strands, and then extending the annealed primers with a DNA polymerase. Multiple cycles of PCR result in an exponential amplification of the DNA template.

Unfortunately, PCR has limitations. These limitations range from 1) the rate of nucleotide incorporation, 2) the fidelity of nucleotide incorporation, 3) the

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length of the molecule to be amplified, and 4) the specificity of the polymerase.

Various methods to improve PCR exist. One approach is to optimize the reaction conditions, e.g., such as the pH, dNTP concentrations, or reaction temperatures. Another approach is to add various chemical compounds, e.g., formamide (Sarkar, G., et al. Nucl. Acids Res. 18: 7465 (1990)), tetramethyammonium chloride, and dimethyl sulfoxide (Chevet et al., Nucl. Acids Res. 23:3343-3344 (1995); Hung et al., Nucl. Acids Res. 18:4953 (1990)) to either increase the specificity of the PCR reaction and/or increase yield. Other attempts include adding various proteins, such as replication accessory factors. Replication accessory factors known to be involved in DNA replication have also increased yields and the specificity of PCR products. For example, *E. coli* single-stranded DNA binding protein, such as RFA, has been used to increase the yield and specificity of primer extension reactions and PCR reactions (U.S. Patent Nos. 5,449,603, and 5.534,407). Another protein, the gene 32 protein of phage T4, appears to improve the ability to amplify larger DNA fragments (Schwartz et al., Nucl. Acids Res. 18: 1079 (1990).

An important modification that has enhanced the ease and specificity of PCR is the use of *Thermus aquaticus* (Taq) DNA polymerase in place of the Klenow fragment of *E. coli* DNA pol I (Saiki et al., Science 230: 1350-1354 (1988)). The use of this thermostable DNA polymerase obviates the need for repeated enzyme additions, permits elevated annealing and primer extension temperatures, and enhances specificity. Further, this modification has enhanced the specificity of binding between the primer and its template. But, Taq

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polymerase has a fundamental drawback because it does not have 3' to 5' exonuclease activity and, therefore, cannot excise incorrect nucleotides added to the ends of the amplified products. Due to this limitation, the fidelity of Taq-PCR reactions typically have suffered. Therefore, those in the field have searched for another thermostable polymerase that has 3' to 5' exonuclease activity.

Polymerases having 3' to 5' exonuclease activity have been found in archaebacteria (archaea). Archaebacteria is a third kingdom, different from eukaryotes and bacteria (eubacteria). Many archaebacteria are thermophilic bacteria-like organisms that can grow in extremely high temperatures, i.e., 100°C. One such archaebacteria is *Pyrococcus furiosus* (Pfu). A monomeric polymerase from Pfu has been identified that has the desired 3' to 5' exonuclease activity and synthesizes nucleic acids of interest at high temperatures (Lundberg et al., Gene 108: 1-6 (1991); Cline et al., Nucl. Acids Res. 24: 3546-3551 (1996) (This polymerase is referred to as Pfu polymerase.)) A second DNA polymerase has been identified in *P. furiosus* which has two subunits (DP1/DP2) and is referred to as pol II. See References 1 and 15. This polymerase may also be enhanced by the accessory factors.

Certain natural proteins exist in archaebacteria, i.e., PEF (polymerase enhancing factors) that exhibit deoxyuracil triphosphatase (dUTPase) activity and that enhance the activity of Pfu polymerase (International Patent Application Publication No. WO 98/42860, published on October 1, 1998). The presence of deoxyuracil-containing DNA in a DNA polymerization reaction inhibits polymerase activity (Lasken et al. (J. Biol. Chem. 271: 17692-17696)).

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Specifically, during the course of a normal PCR reaction, a dCTP may be deaminated into dUTP, thereby introducing a deoxyuridine into the newly synthesized DNA. But, when this newly synthesized DNA is thereafter amplified, the presence of the deoxyuridine inhibits the Pfu polymerase. The archaeal dUTPase (PEF) prevents dUTP incorporation and, thus, avoids the inhibition of the Pfu polymerase. Accordingly, the archaeal dUTPase optimizes the activity of Pfu polymerase.

According to certain embodiments, the invention provides methods of, and materials for, enhancing the polymerase activity of Pfu polymerase. Certain embodiments involve major components of the replication machinery in eukaryotes, e.g.: a helicase enzyme that unwinds the DNA helix and, thereby, provides a single-stranded DNA template; single-stranded DNA binding proteins (RFA) that bind and stabilize the resulting single-stranded DNA template; a "sliding clamp" protein (PCNA) that stabilizes the interaction between the polymerase and the primed single-stranded DNA template and that enhances synthesis of long DNA strands (also known as "processivity"); and a "clamp-loading" protein complex (RFC) that assembles the PCNA protein.

According to certain embodiments, the invention provides novel DNA replication accessory factors which have been isolated and purified from the hyperthermophilic archaeal bacteria *Pyrococcus furiosus*. In certain embodiments, the isolated proteins are thermostable homologues of eukaryotic DNA replication proteins PCNA, RF-C subunits, RFA, and helicases. Recent computer analysis of sequence data do not describe the proteins disclosed

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herein, although sequences that may be homologous to eukaryotic and/or eubacterial replication factors exist (Chedin et al., TIBS 23:273-277 (1998); Egdell and Doolittle, Cell 89: 995-998 (1997); Bult et al., Science 273: 1058-1-73 (1996)).

According to certain embodiments, this invention also involves isolated polynucleotides that encode the replication accessory factors.

In certain embodiments, the polynucleotide may be cDNA, genomic DNA, mRNA, or plasmid DNA.

According to certain embodiments, the invention includes vectors comprising a polynucleotide that encodes a replication accessory factor and host cells comprising such vectors. According to certain embodiments, the invention includes polypeptides expressed in those host cells. Further, this invention provides not only the host cells and their products, but also, the methods of using such host cells to produce the polypeptides of interest.

According to certain embodiments, the invention includes methods of enhancing a nucleic acid polymerase reaction comprising the addition of one or more of the replication accessory factors to the reaction.

In certain embodiments, only one archaeal replication accessory factor will be added into the nucleic acid polymerase reaction. In other embodiments, a combination of factors may be added.

In certain embodiments, an archaeal dUTPase may be combined with one or more of those replication accessory factors to further enhance the polymerase reaction.

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In certain embodiments, this invention also provides methods of synthesizing nucleic acids comprising employing an archaeal polymerase and an archaeal replication accessory factor(s).

According to certain embodiments, the invention includes methods of amplifying nucleic acids of interest comprising employing an archaeal polymerase and an archaeal replication accessory factor(s).

In certain embodiments of the inventive methods, the archaeal polymerase is Pfu polymerase. In certain embodiments of those methods, the archaeal polymerase is combined with another polymerase, such as Taq. In other embodiments of the these methods, an archaeal dUTPase may also be included to enhance polymerase activity.

In certain embodiments of the inventive methods, the archaeal polymerase is *P. furiosus* pol II polymerase.

In certain embodiments, this invention also provides a kit used in the practice of the above-described methods.

In certain embodiments, this invention also provides a kit comprising an archaeal polymerase and at least one archaeal replication accessory factor.

In certain embodiments, those kits would also comprise an archaeal dUTPase and possibly, another polymerase, such as Taq.

Brief Description of the Drawings

The abbreviations used herein for amino acids in the translated protein sequences, which are single letter, and the nucleic acids are those

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conventionally used, as in Stryer et al., Biochemistry, 3rd ed., W.H. Freeman, N.Y. (1988) at the back cover.

Figure 1 illustrates the identification of native PCNA in heparin sepharose fractions. Nucleotide incorporation was measured in the absence of salt to detect polymerase activity ("Pol") or in the presence of NaCl + Pfu DNA polymerase to detect PCNA.

Figure 2 illustrates the identification of native PCNA activity in SDS-PAGE gel slices. An active heparin sepharose fraction was electrophoresed on an SDS-PAGE gel, and slices of the gel were excised and the proteins eluted. The presence of PCNA or polymerase activity was determined as described above in Figure 1 and in the Detailed Description of Embodiments of the Invention.

Figure 3 illustrates the DNA sequence of PCNA.

Figure 4 illustrates the translated protein sequence of PCNA.

Figure 5 illustrates that PCNA enhances the processivity of Pfu DNA polymerase. A 5'-radiolabelled 38 bp oligonucleotide was annealed to single-stranded M13. The template was incubated at 72°C in the presence of cloned Pfu PCR buffer, dNTPs, and either cloned Pfu DNA polymerase or exo$^-$ Pfu DNA polymerase. To certain reactions, ~0.1 or 10 fmoles of PCNA was added. Reactions were allowed to proceed for 1, 5, 10, or 30 minutes, and then stopped in loading buffer. The extension products were electrophoresed on

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CastAway® prepoured 6% (7M urea) gels, and the gels were dried and visualized by autoradiography. The length of the fully extended product is approximately 7 kb.

Figure 6 illustrates the stimulation of TaqPlus® Long DNA polymerase blend (Stratagene) with PCNA. A 23 kb fragment was amplified from genomic DNA using 5U TaqPlus® Long polymerase blend, in the presence of native PEF, a no KCl buffer, and varying amounts of PCNA.

Figure 7 illustrates the stimulation of TaqPlus® Long DNA polymerase blend with PCNA. A 30 kb fragment was amplified from genomic DNA using 5U TaqPlus® Long, in the presence of native PEF, a no KCl buffer, and varying amounts of PCNA.

Figure 8 illustrates the DNA sequence of genomic RFC clones. Genomic sequences encoding the P38 and P55 subunits are located in tandem, respectively. The sequence encoding P38 contains an intein. As used herein, the term "intein" includes, but is not limited to protein splicing elements. These elements are involved in the post-translational processing of pre-proteins. The coding regions of the P38 and P55 subunits are bracketed [ ]. The intein sequence is enclosed in parentheses ( ).

Figure 9 illustrates the translated protein sequence of the genomic RFC clone. The sequence encoding P38 and P55, respectively, are enclosed in parentheses ( ), while the sequence of the intein is

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bracketed [ ]. The * indicates a stop codon.

Figure 10 illustrates the translated protein sequence of recombinant P55 clone.

Figure 11 illustrates the translated protein sequence of recombinant P38 clone.

Figure 12 illustrates a Western blot of immunoaffinity purified native RFC complex using anti-P38 IgG (panel A) or anti-P55 IgG (panel B). Immunoaffinity purification was carried out using rabbit anti P55 IgG as the capture reagent. Fractions are labeled as follows: +, positive control; 0, wash. F20-F34 refer to fractions eluted at pH 2.8 from the column.

Figure 13 illustrates a protein gel of immunoaffinity purified native RFC complex. Immunoaffinity purification was carried out using rabbit anti P55 IgG as the capture reagent. Fractions are labeled as follows: +, positive P38 control; α-P38 (unrelated expt.) or α-P55 column washes (present expt.). F18-F23 refer to fractions eluted at pH 2.8 from the column.

Figure 14 illustrates the ATPase activity of native and recombinant RFC. Positions on the TLC plate containing the released radioactive phosphate were excised and counted in a scintillation counter.

Figures 15 illustrates that native clamp loader further stimulates primer extension by Pfu in the presence of PCNA. Primer extension reactions were carried out as described in the Detailed Description

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of Embodiments of the Invention.

Figure 16 illustrates a cDNA sequence of a clone expressing RFA.

Figure 17 illustrates the translated protein sequence of RFA. The theoretical molecular weight is 41.3 kDa. The native protein may start at the third methionine.

Figure 18 illustrates a gel shift assay that demonstrates single-stranded DNA binding activity of *P. furiosus* RFA. 50 ng of a 38-mer oligo was incubated with *E. coli* SSB (lane 1), water (lane 2), or *P. furiosus* RFA (lanes 3-7) in TE buffer (lanes 1-3), 1x cloned Pfu buffer (lane 4), 50 mM Tris pH 8.5, 25 mM KCl, 2 mM $MgCl_2$ (lane 5), 50 mM Tris pH 8.5, 25 mM KCl, 5 mM $MgCl_2$ (lane 6), or 50 mM Tris pH 8.5, 25 mM KCl, 2 mM $ZnCl_2$ (lane 7). Samples were incubated at 95°C for 10 minutes, followed by 72 °C for 2 minutes prior to loading on a 4-20% acrylamide gradient gel in 1x TBE buffer. Bands were visualized by SYBR green staining and UV illumination.

Figure 19 illustrates an increase in amplification specificity with RFA using cloned Pfu + PEF (Pfu Turbo™ DNA polymerase (Stratagene)) (5.2 kb system).

Figure 20 illustrates an increase in product yield using RFA in combination with cloned Pfu Turbo™ DNA polymerase (2.1 kb system).

Figure 21 illustrates an increase in yield and amplification specificity with RFA and *E. coli* SSB using *Taq* and *Pfu* DNA polymerases (0.5 kb

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system).

Figure 22 illustrates the DNA sequence of recombinant helicase 2. This helicase has demonstrated PCR enhancing activity.

Figure 23 illustrates the DNA sequence of recombinant helicase 3.

Figure 24 illustrates the DNA sequence of recombinant helicase 4.

Figure 25 illustrates the DNA sequence of recombinant helicase 5.

Figure 26 illustrates the DNA sequence of recombinant helicase 6.

Figure 27 illustrates the DNA sequence of recombinant helicase 7.

Figure 28 illustrates the DNA sequence of recombinant helicase dna2. This helicase has demonstrated PCR enhancing activity.

Figure 29 illustrates the translated protein sequence for helicase 2. The theoretical molecular weight is 87.9 kDa + 4.0 kDa (CBP affinity tag).

Figure 30 illustrates the translated protein sequence for helicase 3. The theoretical molecular weight is 100.0 kDa + 4.0 kDa.

Figure 31 illustrates the translated protein sequence for helicase 4. The theoretical molecular weight is 105.0 kDa + 4.0 kDa.

Figure 32 illustrates the translated protein sequence for helicase 5. The theoretical molecular weight is 86.8 kDa + 4.0 kDa.

Figure 33 illustrates the translated protein sequence for helicase 6 + 4.0 kDa (CBP affinity tag).

Figure 34 illustrates the translated protein sequence for helicase 7. The

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theoretical molecular weight is 126.0 kDa + 4.0 kDa.

Figure 35 illustrates the translated protein sequence for helicase dna2 + 4.0 kDa

(CBP affinity tag).

Figure 36 illustrates the ATPase activity of helicases produced by phage induction.

1 microliter of Pfu helicases 3, 4, 5, 6, 7, and 8 (lanes 1-6 respectively), 0.8 units of porcine ATPase (9) or water (10) were incubated with 1 μl of 4.5 micromolar ATP and 1 microCurie of gamma labeled ^{33}P ATP in 1x Optiprime buffer #3 (10mM Tris-HCl (pH 8.3), 3.5 mM $MgCl_2$, 75 mM KCl). The samples were incubated at 72°C for 20 minutes before being spotted on PEI cellulose F. The samples were allowed to dry before the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4. The liquid front was allowed to migrate 5 cm before being removed from the liquid and dried. The samples were exposed to x-ray film for one hour.

Figure 37 illustrates the ATPase activity of helicases produced by IPTG induction of bacterial cultures. 1 microliter of an old lot or new lot of Pfu dna2-like helicase (lanes 1 and 2, respectively), Pfu helicase 2, 3, 4, 5 and 7 (lanes 3-7), water (8), or 0.8 units of porcine ATPase (9) were incubated with 1 μl of 4.5 micromolar ATP and 1 microCurie of gamma labeled ^{33}P ATP in 1x Optiprime buffer #3 (10mM Tris-HCl (pH 8.3), 3.5 mM $MgCl_2$, 75 mM KCl). The samples

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were incubated at 72°C for 20 minutes before being spotted on PEI cellulose F. After drying, the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4. The liquid front was allowed to migrate 4 cm before being removed from the liquid and dried. The samples were exposed to x-ray film for one hour.

Figure 38 illustrates the helicase displacement of bound oligos. Radioactively labeled oligonucleotides with a 3' overhang (A) or a 5' overhang (B) were annealed to M13mp18. The reactions were incubated with 0.5 μl of putative Pfu helicases 3-7 and Pfu helicase dna2 in 50 mM Tris pH 8.5, 25 mM KCl, 5 mM $MgCl_2$ and 5 mM ATP for 30 minutes at 55°C. 1 μl of Pfu helicase 2 was used in an identical reaction. The positive control was generated by thermally melting the annealed oligo prior to loading. The negative control was incubated with water. The samples were run on 4-20% gradient acrylamide gels in 1xTBE. The gels were dried and exposed to x-ray film.

Figure 39 illustrates the enhancement of *Pfu* processivity with *Pfu* PCNA and RF-C.

Figure 40 illustrates the DNA sequence of recombinant helicase 8. Molecular weight

is 82.6 kDa + 4 kDa CBP tag.

Figure 41 illustrates the translated protein sequence of recombinant helicase 8.

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Figure 42 Illustrates the stimulation of nucleotide incorporation by Pfu and P. furiosus pol II DNA polymerases using PCNA. Primer extension reactions were performed at 66-99°C using primed single-stranded M13 DNA. Optimal activity was observed at or above 80°C in the presence of PCNA (no measurements carried out between 80 and 95°C), while reduced activity was observed above 72°C in the absence of PCNA.

Detailed Description of Embodiments of the Invention

The invention provides for isolated and purified polynucleotides that encode novel DNA replication accessory factors from hyperthermophilic archaebacteria. In certain embodiments, the replication accessory factors are from *Pyrococcus furiosus*. These replication accessory factors may be thermostable homologues of the eukaryotic DNA replication proteins PCNA, RFC subunits, RFA, and helicases.

As used herein "isolated and purified polynucleotide" is a nucleic acid, which is substantially separate from at least one other DNA sequence that naturally accompanies the native polynucleotide. Such other DNA sequences may be, e.g., a ribosome, a polymerase, and any other human genomic sequence.

These polynucleotides include RNA, cDNA, genomic DNA, synthetic forms, e.g., oligonucleotides, antisense and sense strands, and may also include chemically or biochemically modified nucleotides, e.g., mutated nucleotides or cys-labeled nucleotides. Recombinant polynucleotides comprising the

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sequences otherwise not naturally occurring are also provided in this invention.

Although polynucleotides having naturally occurring sequences may be employed, such polynucleotides may be altered, e.g., by deletion, substitution, or insertion. One skilled in the art will know appropriate changes in the sequence that will encode proteins that retain biological activity. In certain preferred embodiments, polynucleotides may be changed to encode different conservative amino acid substitutions. Conservative amino acid substitutions include, but are not limited to, a change in which a given amino acid may be replaced, for example, by a residue having similar physiochemical characteristics. Examples of such conservative substitutions include, but are not limited to, substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another; substitutions of one polar residue for another, such as between Lys and Arg, Glu and Asp, or Gln and Asn; or substitutions of one aromatic residue for another, such as Phe, Trp, or Tyr for one another. Other conservative substitutions, *e.g.*, involving substitutions of entire regions having similar hydrophobicity characteristics, are well known. See Biochemistry: A Problems Approach, (Wood, W.B., Wilson, J.H., Benbow, R.M., and Hood, L.E., eds.) Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA (1981), page 14-15.

cDNA or genomic libraries of various types may be screened as natural sources of the polynucleotides of the present invention, or such nucleic acids may be provided by amplification of sequences that exist in genomic DNA or other natural sources, e.g., by PCR. See, e.g., PCR Protocols: A Guide to Methods and Application, Innis, M., et al., eds., Academic Press: San Diego

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(1990). Genomic polynucleotides encoding the archaeal replication accessory factors may contain additional non-coding bases, or inteins, and one skilled in the art would know how to obtain such polynucleotides. One way to obtain genomic DNA sequences is by probing a genomic library with all or part of a known DNA sequence. The obtained genomic DNA sequence should encode functional proteins.

In certain embodiments of this invention, the nucleic acid sequences of the isolated polynucleotides encoding the replication accessory factors have been obtained and may be used for various purposes. In certain embodiments, the invention includes isolated and purified polynucleotides that encode the following: an archaeal PCNA, an archaeal RFC subunit P38 protein, archaeal RFC subunit P55, archaeal RFC subunit P98, archaeal RFA, and various archaeal helicases. According to certain embodiments, the invention includes eight different helicases that exist in Pfu, i.e., helicase 2 to 8, and helicase dna2. According to certain embodiments, the polynucleotide sequences are set forth in Figures 3, 8, 16, 23 to 28, and 40.

As used herein, the term "PCNA" may also be referred to as a "clamp" or a "sliding clamp" protein, in view of its role in clamping the DNA polymerase to the DNA template in eukaryotes.

In certain embodiments, the term "RFC subunits" includes, but is not limited to, proteins of about 55 kDa and about 38 kDa in molecular weight or subunits having the amino acid sequence set forth in Figure 10 and Figure 11, respectively. These subunits are referred to herein as "P55" and "P38." These

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subunits are part of a complex having one large subunit and at least one small subunit. P55 is considered a large subunit and P38 a small subunit.

This invention further provides for isolated and purified polynucleotides that encode amino acid sequences for various replication accessory factors, such as an archaeal PCNA, archaeal RFC subunit P38 protein, archaeal RFC subunit P55, archaeal RFA, and various archaeal helicases. According to certain embodiments, the amino acids sequences of those polynucleotides are set forth in Figures 4, 9 to 11, 17, 29 to 35, and 41.

These polynucleotides described herein also include nucleic acid sequences that encode for polypeptide analogs or derivatives of the various archaeal replication accessory factors, which differ from naturally-occurring forms, e.g., deletion analogs that contain less than all of the amino acids of the naturally-occurring forms, substitution analogs that have one or more amino acids replaced by other residues, and addition analogs that have one or more amino acids added to the naturally-occurring sequence. These various analogs share some or all of the biological properties of the archaeal replication accessory factors. As noted above, one skilled in the art will be able to design suitable analogs. In certain preferred embodiments, conservative amino acid substitutions will be made. In certain embodiments, the analogs will be 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to the naturally-occurring sequence.

Percent identity involves the relatedness between amino acid or nucleic acid sequences. One determines the percent of identical matches between two

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or more sequences with gap alignments that are addressed by a particular method. The percent identity may be determined by visual inspection and mathematical calculation. Alternatively, the percent identity of two nucleic acid sequences can be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Other programs used by one skilled in the art of sequence comparison may also be used.

In certain embodiments, nucleic acids may be those that hybridize under moderately or highly stringent conditions to the complement of naturally-occurring encoding nucleic acids or to nucleic acids that encode proteins having naturally-occurring amino acid sequences. As used herein, conditions of moderate stringency can be readily determined by those having ordinary skill in the art based on, for example, the length of the DNA. The basic conditions are set forth by Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989), and include

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use of a prewashing solution for the nitrocellulose filters 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridization conditions of about 50% formamide, 6X SSC at about 42°C (or other similar hybridization solution, such as Stark's solution, in about 50% formamide at about 42°C), and washing conditions of about 60°C, 0.5X SSC, 0.1% SDS. Conditions of high stringency can also be readily determined by the skilled artisan based on, for example, the length of the DNA. Generally, such conditions are defined as hybridization conditions as above, and with washing at approximately 68°C, 0.2X SSC, 0.1% SDS. The skilled artisan will recognize that the temperature and wash solution salt concentration can be adjusted as necessary according to factors such as the length of the probe.

In certain embodiments, polynucleotides may have sequences different from the naturally-occurring nucleic acid sequence in view of the redundancy in the genetic code, especially if the amino acid sequences are known. Various codon substitutions may be introduced to produce various restriction sites or to optimize expression in a particular system.

The polynucleotides used in this invention will usually comprise at least about 15 nucleotides. In certain embodiments, the number of nucleotides is the minimal length required to express a biologically active replication accessory factor or, to probe for nucleic acid sequences encoding a replication accessory factor, or for nucleic acid priming.

Techniques for manipulating polynucleotides are described generally in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, eds. Sambrook et al. (Cold Spring Harbor Laboratory Press 1989)). Reagents useful

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in applying such techniques, e.g., restriction enzymes, are widely known in the art and commercially available from vendors such as Stratagene

These polynucleotides may be used as nucleic acid probes and primers. Such probes and primers would be useful in screening for other archaeal replication accessory factors or screening other species for homologous replication accessory factors. The probe or primer may comprise an isolated nucleic acid, and may include a detectable label, such as a reporter molecule.

In certain embodiments of this invention, one may also want to generate viral or plasmid DNA vectors using the polynucleotides disclosed herein. The contemplated vectors include various viral vectors. Some commonly used examples are, but are not limited to, plasmids, bacteriophages, retroviruses, and adenovirus. Such vectors may be coupled with nucleic acids that encode an origin of replication (ORI) or autonomously replicating sequence (ARS), expression control sequences, e.g., promoter and enhancer sequences, and protein processing information sites, such as RNA splice sites, polyadenylation sites, ribosome-bind sites, and mRNA stabilizing sequences. Such vectors and the methods used in constructing them are well known in the art. See, e.g., Sambrook et al: *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, Cold Spring Harbor Laboratory Press, (1989); Pouwels et al., Cloning Vectors: A Laboratory Manual, Elsevier, New York, (1985); "Gene Expression Technology" Methods in Enzymology, v. 185, D.V. Goeddel, ed. Academic Press Inc., San Diego, CA (1990); and "Viral Vectors: Gene Therapy and Neuroscience Applications" (Kaplitt, M.G., and Loewy, A.D., eds.) Academic Press, San Diego,

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CA (1995).

These polynucleotide may include the incorporation of codons "preferred" for expression of the polynucleotides in selected nonmammalian hosts, e.g., prokaryotic or non-mammalian eukaryotic host cells.

Vectors may be used to introduce the polynucleotides of this invention into a host cell. Typically, these vectors also include transcription and translational initiation regulatory sequences operably linked to the polynucleotide that encodes an archaeal replication accessory factor. These vectors would facilitate the production of such a factor in a host cell.

In certain embodiments, to produce the replication accessory factor encoded by the polynucleotide, an appropriate promoter and compatible host cell may be chosen. Examples of compatible cells lines and expression vectors are well known in the art. Certain well known host cells are prokaryotes like E. coli, and B. Subtilis. In a prokaryotic host cell, such as *E. coli*, a polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant polypeptide. Examples of eukaryotic host cells are yeast, fungi, plant, insect, amphibian, avian, or mammalian cells. See, e.g., "Gene Expression Technology" Methods in Enzymology, v. 185, D.V. Goeddel, ed. Academic Press Inc., San Diego, CA (1990).

In certain embodiments, one may employ a selectable marker in the host cell system or vector such that transformed cells are be easily detectable. In certain embodiments, such markers are detectable after the cells have been

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transformed. An example includes, but is not limited to, antibiotic resistance.

Those skilled in the art will be able to construct suitable expression systems in suitable host cells, especially in view of the many publications, including manuals, that discuss such information.

This invention provides a method for producing archaeal replication accessory factors by expressing a vector that comprises a polynucleotide that encodes a replication accessory factor in a suitable host cell and purifying the expressed product. Techniques using such host cells to express such polynucleotides are well known in the art. See, e.g., Sambrook et al. (1989).

This invention also provides recombinant protein produced by the above-described method.

The invention also provides isolated and purified archaeal replication accessory factors including, but not limited to, archaeal PCNA, archaeal RFC-P38, archaeal RFC-P55, archaeal RFA, and archaeal helicases, e.g., dna2 and helicases 2 to 8.

In certain embodiments, these accessory factors have part or all of the primary structural conformation and one or more of the biological properties of a replication accessory factor.

As used herein, "isolated and purified protein" describes a replication accessory factor separate from at least one other protein that naturally accompanies the protein.

In addition to naturally-occurring allelic forms of the replication accessory factors, this invention also includes polypeptide analogs or fragments. One of

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skill in the art can readily design nucleic acid sequences that express such analogs or fragments of the replication accessory factors. For example, one may use well-known site-directed mutagenesis techniques to generate polynucleotides encoding such analogs or fragments. Those analogs and fragments may have one or more of the biological functions of the naturally-occurring replication accessory factor.

Further, this invention provides a composition comprising at least one archaeal replication accessory factor for use in nucleic acid polymerase reactions. As used herein "nucleic acid polymerase reactions" includes, but is not limited to, PCR-based reactions that may include site-directed mutagenesis, amplification, and synthesis of nucleic acid of interest.

In certain embodiments of the invention, the composition further comprises at least one polymerase. Such a polymerase may include, but would not be limited to, Pfu polymerase, *P. furiosus* pol II polymerase, and/or Taq polymerase.

In certain embodiments, the polymerase is an archaeal polymerase. The archaeal DNA polymerase may be obtained from archaea such as *Pyrococcus species GB-D, Pyrococcus species strain KOD1, Pyrococcus woesii, Pyrococcus abysii, Pyrococcus horikoshii, Pyrodictium occultum, Archaeoglobus fulgidus. Sulfolobus solfatanicus, Sulfolobus acidocaldarium. Thermococcus litoralis, Thermococcus species 9 degrees North-7. Thermococcus species JDF-3, Thermococcus gorgonarius, Methanobacterium thermoautotrophicum. Methanococcus jannaschii, Methanococcus voltae, Thermoplasma acidophilum.*

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Related archaea from which the archaeal DNA polymerase may be obtained are also described in Archaea: A Laboratory Manual (Robb, F.T and Place, A.R., eds, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1995).

According to certain embodiments, the archael polymerase is related to Pfu (pol I) or *P. furiosus* pol II. Commercial enzymes that are related to Pfu (pol I) that are likely to function with the *P. furiosus* replication factors are; KOD (Toyoba), pfx (Life Technologies Inc.), Vent (New England Biolabs), Deep Vent (New England Biolabs), and Pwo (Roche Molecular Biochemicals). Archaea which contain genes that exhibit DNA sequence homology to *P. furiosus* pol II subunits are described in references (Makinjemi, M. et al. (1999) Trends in Biochem., Sci. 24:14-16; Ishino et al. (1998) J. Bacteriol., 180 2232-6).

In certain embodiments the archaeal factors are used with a thermostable eubacterial polymerase, or are used with a mixture of a eubacterial and archaeal polymerase. Thermostable eubacterial polymerases may be related to the pol I, pol II, or pol III class of DNA polymerases. Thermostable pol I DNA polymerases have been described in *Thermus species (aquaticus, flavus, thermohilus HB-8, ruber, brokianus, caldophius GK14, Filiformis), Bacillus species (stearothermophilus, caldotenex YT-G, caldovelax YT-F), and Thermotoga maritima.* Commercial enzymes that are related to eubacterial pol I enzymes include Taq (Stratagene) Tth (Perkin Elmer), Hot Tub/Tfl (Amersham), Klen Taq (Clone Tech), Stoffel fragment (Perkin Elmer), UlTma (Perkin Elmer), DynaZyme (Finnzymes), Bst (New England Biolabs), and Bca (Panvera). Thermostable pol III DNA polymerases have been described in *Thermus aquaticus (Huang, et al.*

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(1999) J. Mol. Evol. 48:756-69) and Thermus thermohilus (reference #13), but could be obtained from other thermophilic eubacteria. Additional thermophilic eubacteria are described in the reference: "Thermophilic Bacteria," Kristjansson, J.K., CRC Press, Inc., Boca Raton, Florida, 1992.

In certain embodiments, to further enhance the nucleic acid polymerase reaction, the invention may also include an archaeal dUTPase (PEF) in the composition.

Unlike current methods of enhancing nucleic acid polymerase reactions, this invention also discloses a method of enhancing nucleic acid polymerase reactions comprising employing a composition comprising at least one archaeal replication accessory factor. Such method will enhance the synthesis, amplification, or mutagenesis of nucleic acids of interest.

According to certain embodiments, the accessory factors enhance any polymerization reaction. Polymerization reactions include primer extension reactions, PCR, mutagenesis, isothermal amplification, DNA sequencing, and probe labeling. Such methods are well known in the art. Enhancement may be provided by stimulating nucleotide incorporation and reducing dissociation of the polymerase from the template. In addition, enhancement may be provided by reducing impediments in the nucleic acid templates, such as secondary structure and duplex DNA. Overcoming or improving such impediments through the addition of accessory factors like RFA and helicase, can allow polymerization reactions to occur more accurately or efficiently, or allow the use of lower denaturation/extension temperatures or isothermal temperatures. In addition,

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according to certain embodiments, RFA and helicase may provide additional benefits in non-polymerizing applications which require single-stranded nucleic acids. For example, RFA may improve the specificity of protein/nucleic acid interactions.

According to certain embodiments, PCNA alone or with other accessory factors may enhance exonuclease reactions carried out by the 3'-5' exonuclease activity of Pfu. Exonuclease reactions are used to prepare long single-stranded DNA templates. Enhancement may be provided by reducing dissociation of the polymerase from the template.

In addition to enhancing polymerization and exonuclease reactions, PCNA is

expected to enhance repair processes that are mediated by Pfu or P. furiosus pol II, and that typically require additional repair proteins, such as Fen-1 and ligase.

According to certain embodiments, PCNA can also be used to enhance processes that are based upon the binding of nucleic acid sequences to complementary nucleic acid strands. For example, hybridization of labeled probes to complementary DNA or RNA strands is used in such methods as library screening, Southern blotting, Northern blotting, chip-based detection strategies, and Q-PCR detection strategies (e.g.,

molecular beacon hybridization probes). Such methods are well known in the art. Increasing the stability of annealed probes by the addition of PCNA may enhance specificity of hybridization reactions by allowing more stringent

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hybridization conditions to be used, such as higher temperature and/or lower ionic strength. Increasing the stability of primer/template interactions may also allow one to carry out more efficient polymerization reactions using RNA polymerases, reverse transcriptases and other nucleic acid polymerizing enzymes.

This invention also provides kits for nucleic acid polymerase reactions that include at least one archaeal replication accessory factor, and possibly other proteins or compounds known to enhance such reactions. In certain embodiments, the kits may also include one or more polymerases.

In certain embodiments, the kits are for synthesizing, amplifying, or mutagenizing nucleic acids of interest.

Certain embodiments of the invention are described in the following examples. However, these examples are offered solely for the purpose of illustrating the invention, and should not be interpreted as limiting the invention to these examples.

Experiments

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Methods

1. Production of Accessory Factors from *Pyrococcus furiosus*

A. DNA Sequence Identification/PCR Primers.

The DNA sequences surrounding the DNA sequences of interest were examined for likely start and stop codons. The majority of DNA sequences of interest were identified in archaeal genome databases (Pyrococcus horekoshi, Pyrococcus furious, Methanococcus jannaschii, Methanobacterium thermoautotrophicum), through similarity to eukaryotic genes encoding replication factors of interest (see reference No. 5). Also, the oligonucleotide sequence for PCNA was identified by N-terminal peptide sequencing of a protein isolated from a native protein preparation (see below). Table 1 below lists PCR primers used to amplify the genes and to produce ends that can be modified to produce cohesive ends with a cloning vector. The sequence corresponding to the vector sequence is underlined.

Table 1

Gene Name	*Forward Primer*	*Reverse Primer*
RFC P98/P38	GACGACGACAAGATGAGCGAAGAGAT TAGAGAA	GGAACAAGACCCGTTCACTTCTTCC CAATTAGGGT
RFC P55	GACGACGACAAGATGCCAGAGCTTCC CTGGGTA	GGAACAAGACCCGTTCACTTTTTAA GAAAGTCAAA
PCNA	GACGACGACAAGATGCCATTCGAAATA GTCTTTG	GGAACAAGACCCGTTCACTCCTCAA CCCTTGGGGCTA
RFC P98Intein Deletion	ACTACAGCGGCTTTGG	CTTTCCGACACCAGGG

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Primers*		
RFA	GACGACGACAAGATGATCATGAGTGCA TTTACAAAAGAAGAAATAATC	GGAACAAGACCCGTTCACATCACCC CCAATTCTTCCAATTCCC
dna2 helicase	GACGACGACAAGATGAACATAAAGAGC TTCATAAACAGGCTT	GGAACAAGACCCGTTCAAATGCTAT CCTTCGTTAGCACAACATA
Helicase 2	GACGACGACAAGATGATTGAGGAGCT GTTCAAGGGATTAGAGAGTGAAAT	GGAACAAGACCCGTTCATCTTTTTA CGGCAAATGCGAATTCTTCTCCCTT
Helicase 3	GACGACGACAAGATGTTAATAGTTGTA AGACCAGGAAGAAAAAAGAATGA	GGAACAAGACCCGTTCATCGTCTCT CACCCTTCAAAATTTTTCCTTCTTC
Helicase 4	GACGACGACAAGATGCACATATTGATA AAAAAGGCAATAAAAGAGAGATT	GGAACAAGACCCGTCTATTCCCAAA ACTTTCTAGTTTGGATGTAGTGTTT
Helicase 5	GACGACGACAAGATGTTATTAAGGAGA GACTTAATACAGCCTAGGATAT	GGAACAAGACCCGTCTACTCCTCAT CCTCTATATATGGGGCAGTTATTA
Helicase 6	GACGACGACAAGATGCTCATGAGGCC AGTGAGGCTAATGATAGCTGATG	GGAACAAGACCCGTCTAGCTTAACT TAAGTAAATGCCTATCTTTCTTCT
Helicase 7	GACGACGACAAGATGATCGAAGGTTAC GAAATTAAACTAGCTGTTGTAAC	GGAACAAGACCCGTTCAAAAACCTT TCCCAGGTATGCGGGGGTCGCT
Helicase 8	GACGACGACAAGATGAGGGTTGATGA GCTGAGAGTTGATGAGAGGATA	GGAACAAGACCCGTTCAAGATTTGA GAAAGTAATCAAGGGTACTTTTTCT

B. PCR Amplification.

1) Procedure.

DNA sequences for PCNA, RFC P98/38, RFC P55, RFA, and helicases dna2 and helicases 2-8 were amplified with various PCR enzymes and polymerase blends using the primers in Table 1. The optimal amplification procedure is described below.

PCR Reaction Mixture:

10 μl 10x cloned Pfu buffer (Stratagene)
0.8 100mM dNTP
3 μl mixed primers (100 ng/μl of each primer)

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1.5 μl PfuTurbo DNA polymerase (2.5 U/μl) (Stratagene)
1 μl genomic or plasmid DNA (approximately 100 ng/μl)
83.7 μl H_2O

2) Temperature Cycling.

Samples were amplified in a RoboCycler® temperature cycler (Stratagene). The extension time used was proportional to the amplification product size. Optimally, the extension time is 2 minutes per kilobase. The annealing temperature depended on the length and composition of the primers, which were usually designed with a Tm (melting temperature) between 50°C-60°C. A standard temperature cycling scheme is listed below:

95°C 1 minute 1 cycle

The following three steps are performed sequentially and are repeated for 30 cycles:

95°C 1 minute

50°C 1 minute

72°C 2 minutes/kb of target

C. Cloning of PCR Products.

1) PCR Product Purification.

Three to 10 PCR reactions were generated for each DNA sequence. The PCR products were combined and purified with Stratagene's StrataPrep® PCR

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purification kit according to its instructions. The purified products were examined on agarose gels (1% agarose/1XTBE) to verify product size and homogeneity. The gels were stained with ethidium bromide and visualized. If any spurious bands were present, products of the correct size were isolated with Stratagene's StrataPrep® DNA gel extraction kit.

2) Insert Preparation (Ligation Independent Cloning (LIC) Method).

35 μl of purified PCR products were added to reactions containing:

5 μl 10x cloned Pfu buffer
1 μl 25 mM dATP
1 μl cloned Pfu DNA polymerase (2.5 u/μl)

and the volume of each reaction was brought to 50 μl with 8 μl H_20. The samples were incubated for 20 minutes at 72°C in the RoboCycler® temperature cycler. This process allows the 3' to 5' exonuclease activity of the polymerase to remove bases at the 3' ends of the PCR products until a dA nucleotide is encountered. The presence of dATP in the reaction prevents further exonucleolytic degradation of the PCR product and the exposed 5' overhangs anneal precisely with the pCALnEK vector. This vector is available commercially from Stratagene and is used to produce annealing termini complimentary to the prepared insert.

3) Annealing.

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The treated PCR products were allowed to come to room temperature before 40 μl of each prepared insert was added to separate tubes containing 40 ng of the LIC-ready pCALnEK vector. Samples were mixed and left to anneal for 16 hours at room temperature.

D. Transformation.

The annealed vector/inserts were transformed into competent cells, namely, Stratagene's Epicurian Coli® XL10-Gold® ultracompetent cells, and selected on LB- ampicillin plates. LB media is a commonly used reagent that would be understood by those practiced in the arts. LB amp plates are made by mixing:

10g	NaC1
5g	Yeast Extract
10g	Tryptone
10g	Agar.

Add H_20 to a final volume of 1 liter. Autoclave. Cool to 55°C. Add ampicillin to a concentration of 100 micrograms per ml. Mix well. Pour about 25 ml per plate.

Supercoiled DNA was isolated from the transformants using the instructions recommended in Stratagene's StrataPrep® Plasmid Miniprep Kit. The plasmids were used to transform BL21(DE3) CodonPlus® or BL2I (DE3)

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pLysS (Stratagene) cells. These cells were again selected on LB-ampicillin plates.

E. Preparation of Recombinant Protein.

1) Bacterial Expression of Recombinant Proteins.

The transformants were grown up in multiple liter batches from overnight cultures preferably in LB media supplemented with Turbo Amp™ antibiotic (Stratagene) at 100 μg/_l at 37°C with moderate aeration. When the cultures reached OD_{600} readings of 0.6 to 1.0, the cells were induced with 1 mM IPTG (Stratagene) and incubated in the same manner for 2 hours to overnight (16 hours). Induction causes the vector to produce recombinant protein with a calmodulin binding peptide (CBP) amino tag. The induced cells were collected by centrifugation and stored at -20°C.

Some helicase clones appeared to be unstable in BL21(DE3) cells. Supercoiled plasmids containing these helicases were transformed into BL21 CodonPlus® cells (Stratagene) and induced with bacteriophage Lambda CE6 (Stratagene) which contains the T7 RNA polymerase gene that provides significant production of protein in BL21 cells. Five to ten ml of $3x10^{10}$ plaque forming unit (pfu)/ml lambda CE6 stock (made according to provided instructions in Lambda CE6 Induction Kit (Stratagene)) was used to induce 500 ml cultures for four hours at 37°C with moderate aeration.

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2) Purification of Recombinant Proteins.

Frozen cells were resuspended to an approximate concentration of 0.25 g/ml in buffers identical or similar to calcium binding buffer: 50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate and 2 mM $CaCl_2$. Cell suspensions were subjected to sonication three times with a Bronson Sonifier 250 at a duty cycle of 80% and an output level of 5 for 45 seconds. The suspensions were left on ice to cool between sonication events. The lysate was cleared by centrifugation at 26,890 g.

The cleared lysates were added to a milliliter of calmodulin agarose (CAM agarose), equilibrated in buffer. Recombinant protein was bound to the CAM agarose (Stratagene) via the CBP tag by incubation with gentle agitation at 4°C. After two hours, the reactions were centrifuged at 3000 g for 5 minutes to collect the CAM agarose and recombinant protein. The lysate supernatant was removed and the CAM agarose was washed at least once by resuspending the resin in 50 ml of calcium binding buffer followed by collection of the CAM agarose by centrifugation as described above. The CAM agarose was transferred to a disposable 15 ml column, packed, and then washed with at least 50 ml of the calcium binding buffer. Recombinant proteins were eluted from the column by using a buffer similar or identical to 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EGTA. Similar buffers may include 2mM EGTA and the salt requirement may vary from protein to protein. Certain proteins required elution at higher ionic strength using a buffer with 1M NaCl. Proteins were evaluated for

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size and purity using Tris-Glycine 4-20% acrylamide gradient gels (Novex) with an SDS loading dye (Novex). Gels were stained with silver or Sypro Orange (Molecular Probes).

F. Removal of Intein Sequence from Recombinant RFC P98 clone.

By alignment to eukaryotic RFC sequences, it was observed that the RFC P98 clone contained an intein sequence. In Figure 8, the intein sequences are marked in parentheses, and correspond to nucleotides 374 to 2028. Upon post-translational excision of the intein, the predicted size of the RFC subunit would decrease from 98 kDa to 38 kDa, and hence this RFC subunit is referred to as P38. To improve expression of recombinant P38 from the RFC P98 clone, the intein was removed by making 5' phosphate modified oligonucleotides that primed immediately upstream and downstream to the sequence coding for the intein termini (see primer sequence in table above, marked as *). The oligos were designed to have their 3' termini pointing away from the intein (inverse PCR). By using the RFC P98/pCALnEK plasmid as a template, all the plasmid/insert sequence was amplified with the exception of the intein. The PCR product was purified with StrataPrep PCR Purification Kit and ligated at room temperature for 16 hours prior to transformation, as described in section 1(D).

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2. Protein Analysis Techniques.

A. Preparation of Antibodies.

Rabbit sera containing specific IgG was prepared by immunizing rabbits with the recombinant accessory factors. CBP-tagged fusion proteins were used to immunize 1-2 New Zealand white rabbits using the following immunization schedule: each rabbit was injected with 90-200 μg CBP-tagged fusion protein (as obtained from section 1(E)(2) above) in Complete Freund's Adjuvant (CFA), inject each rabbit with a booster 18 days later including 45-100 μg CBP-tagged fusion protein in incomplete Freund's adjuvant (IFA); inject each rabbit with a second booster of IFA 39 days later; and obtain the first serum sample 45 days later and at various times thereafter.

B. SDS-PAGE.

Native and recombinant protein samples were analyzed on 4-20% acrylamide/2.6% bis-acrylamide Tris-Glycine gels (NOVEX), stained with either silver stain or Sypro orange (Molecular Probes). Protein concentrations were determined relative to a bovine serum albumin (BSA) standard (Pierce), using Pierce's Coomassie Blue Protein assay reagent or by comparisons of relative staining intensities on SDS-PAGE gels.

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C. Western Blot.

Protein samples were transferred from SDS-PAGE to nitrocellulose by electroblotting using standard techniques. The blots were blocked with 1% Blotto/TBS (instant milk in tris buffered saline) for 1 hour at room temperature, followed by incubation with immunized rabbit sera which had been diluted 1:500 or 1:1000 (1 hour). Blots were washed 3 times with TBS containing 0.01% Tween 20. The blots were then incubated for 0.5-1 hour with alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 1:500 or 1:1000 in TBS-0.01% Tween 20. Finally, the blots were washed as before and then incubated in color development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/ml NBT, and 0.15 mg/ml BCIP) for approximately 1-10 minutes. The enzyme reaction was stopped and the membrane was washed five times with deionized water.

D. Amino acid sequence analysis.

Protein samples were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad). Blots were sent to Beckman Research Institute-City of Hope (Duarte, CA) for N-terminal sequence analysis.

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3. Isolating DNA encoding Recombinant RFC from a Genomic Library (Alternative Method)

A *Pyrococcus furiosus* genomic library was plated on XL1-Blue MRF' *E. coli* (Stratagene) at a density of approximately 2000 plaques per plate. Duralose filters (nitrocellulose on nylon backing) were used to take replicate lifts from each plate. While the first filter was on the plate, orientation marks were made by stabbing a needle through the filter and into the plate. The orientation marks were marked in pen on the back of the plate before the filter was removed. The filter lifts were treated as follows:

1.5-2.0 minutes	1.5 M NaCl, 0.5 M NaOH
2 minutes	0.5 M Tris (pH 8.0), 1.5 M NaCl
30 seconds	2xSSC, 0.2 M Tris (pH 7.5)

After treatment, the filters were partially dried until they were still damp, but no standing water was visible. The DNA was fixed onto the filters by UV crosslinking with the Stratalinker (Stratagene) set to the "Autolink" format according to the instructions.

The filters were prehybridized in 15 ml of:

5x SSC
40 mM $NaPO_4$ pH (6.5)
5x Denhardt's
5% Dextran Sulfate
50% Formamide
0.1 mg/ml Salmon sperm DNA (Boiled separately and added immediately prior to use)

Prehybridization was carried out at 42ºC for approximately 2 hours.

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Probe was generated from a 200 bp PCR product amplified from *Methanococcus jannaschii* genomic DNA using the following primers:

Oligo # 576: GAT GAA AGA GGG ATA GAT

Oligo # 577: ATC TCC AGT TAG ACA GCT

These PCR primers were designed to anneal to regions flanking a 200 bp sequence of the *Methanococcus jannaschii* RF-C gene that exhibits 52% amino acid identity to the RF-C gene from human. (See Section 2 under Results below).

The PCR product was purified from free primers, buffer and nucleotides and 50 ng of the product was labeled with ^{32}P-αdATP using the Stratagene Prime-It II Random Primer Labeling kit. The probe was purified from free nucleotides before being boiled for five minutes and added to the prehybridization reaction. The total probe was calculated to be 20 million cpm. Hybridization was allowed to continue overnight at 42ºC before the hybridization solution was removed and the filters were washed four times with 0.1x SSC, 0.1% SDS at 60ºC (very stringent conditions). The filters were exposed to X-ray film overnight and 20 primary isolates with strong signals on both replicate filters were picked.

Three primary isolates were diluted, plated, and screened again using the same method described above. Two filters produced positive lambda clones. Bluescript plasmid clones were excised from the lambda clones in SOLR cells

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(Stratagene) according to the manufacturer's instructions. The clones had inserts sizes of 8 kb and 10 kb. These plasmid clones were cut with Hind III, blotted, and probed with the original 200 bp PCR product discussed above. One positive truncated clone was isolated and sequenced from each end of the insert. The sequence showed two RF-C sequences, specifically, the C-terminus of one sequence, and the N-terminus of another.

4. Production of Accessory Factors from Native Sources

A. *P. furiosus* extract.

Fermentation of *P. furiosus* DSM 3638 cells was carried out using the procedure described in Archaea: A Laboratory Manual, Robb, F.T. (editor-in-chief), Cold Spring Harbor Press, CSH, NY 1995. The cell paste was resuspended in lysis buffer (50 mM Tris-HCl (pH 8.2), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM beta-mercaptoethanol (_-ME), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 _g/ml aprotinin) and lysed in a French press, and then the lysate was sonicated and centrifuged.

B. Column Chromatography.

The supernatant was chromatographed on a Q-Sepharose Fast Flow column (Pharmacia), equilibrated in 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, and 10 mM _-ME. Follow-through fractions were collected, adjusted to pH 7.5, and then loaded onto an SP Sepharose Big Bead column (Pharmacia), and

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equilibrated in buffer A (50mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.1% (v/v) Igepal CA-630, and 0.1% (v/v) Tween 20). The column was eluted with a 0-0.25 M KCl gradient/Buffer A. Fractions containing DNA polymerase activity were dialyzed, and then applied to a Heparin Sepharose CL-6B column (Pharmacia), and equilibrated in Buffer B (same as buffer A, except pH 8.2). The column was eluted with a 0-0.3 M KCl gradient/Buffer B. Fractions exhibiting polymerase enhancing activity (nucleotide incorporation) (using the assay described below in Section 5(B)), or immunocrossreactivity (using the Western Blot described in Section 2(C) above) were identified. Native PCNA was further purified by excising the protein from SDS-PAGE gels.

C. Immunoaffinity chromatography.

Immunoaffinity columns were prepared using the commercial kit from Pierce (ImmunoPure Plus Cat# 44893), following the manufacturer's method. Two milliliters of serum, mixed with 2 milliliters of kit loading/binding buffer, was used to prepare each column. Before using the column, the column and buffers were allowed to warm to room temperature.

1) Preparation of extract.

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0.5 g frozen *P. furiosus* cells were used for each column. The cells were lysed in 2 ml lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, and 1 mM DTT), and sonicated twice for 2 minutes on ice. The cells were spun at 26,890 g for 15 minutes and the supernatant was recovered. The lysate was mixed with an equal volume of binding buffer (10 mM Tris pH 8.0, 50 mM KCl, 0.1% Tween 80). The lysate was precleared by incubation with 4 ml of protein A beads (Pierce cat # 20333) and equilibrated with binding buffer. The slurry was incubated at 4ºC for 1 hour with agitation. The precleared extract was recovered by packing the beads into a disposable column and the flow-through was collected. The column was washed with 2 ml of binding buffer and the wash was collected and pooled with the flow-through fraction. The final volume of pretreated lysate is about 6 ml. In some cases, the lysate was then run over a pre-bleed rabbit IgG control column to further clean up the lysate.

2) Immunoaffinity chromatography.

The column was equilibrated with 15 ml binding buffer (10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.1% Tween 80), and then, the 6 milliliters of precleared lysate was applied to the column. The column was washed with 10 milliliters binding buffer, followed by 10 milliliters wash buffer (10 mM Tris pH 8.0, 500 mM KCl, 0.1% Tween 80). Specific proteins were eluted with seven 1 ml washes of elution buffer (0.1 M glycine pH 2.8). One ml fractions were collected. To each collection tube, was added 50 µl 1M Tris pH 9.5 to raise the pH of the eluates as

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they are collected. After eluting the protein of interest, the column was washed with 4 ml of 1M Tris pH 8.0 and then 15 milliliters of binding buffer.

5. Biochemical Assays

A. Primer Extension Assay.

The *Pyrococcus furiosus* accessory proteins were tested for their ability to stimulate the processivity of cloned Pfu polymerase activity on primed single-stranded M13 DNA. One version of this assay provided for detecting extension products under non-denaturing conditions using ethidium bromide staining. For this assay, a reaction cocktail was made containing:

5 μg/ml	single-stranded M13 mp 18(+) strand DNA (Pharmacia cat# 27-1546-01)
275 ng/ml	40-mer primer (5' GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GTG C 3')
200 μMeach	dNTP
1X	cPfu buffer
	water to 20 μl.

Single stranded M13 DNA was mixed with primer, buffer, and water. The mix was heated to 95ºC for 2 minutes and then cooled to room temperature. The rest of the reaction components were added. Each 20 μl reaction contained 0.05 units of cloned Pfu polymerase and varying amounts of PCNA and RFC. For assessing *P. furiosus* RF-C enhancement, assays contained 0.025 μl of *P. furiosus* PCNA (about 1 ng), and varying amounts of native *P. furiosus* RF-C. The reactions were incubated at 72ºC for 15 minutes. 2 μl DNA loading dye

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(50% glycerol, 1xTBE, .05% bromphenol blue + .05% xylene cyanol) was added to each sample and 15 µl of sample with dye was loaded in each well of a 1% agarose gel (Reliant, FMC cat# 54907). The gel was stained with ethidium bromide. The double-stranded M13 can be seen as a brightly staining product that migrates higher than a 12 kb marker similar to the position where a double-stranded M13 DNA control migrates. In this assay, one looks for an increase in the size of products synthesized when PCNA or PCNA + RFC are added to Pfu. Ethidium bromide staining is proportional to the amount of double-stranded DNA produced from primed single-stranded M13.

A second version of this gel-based assay allows detection of radiolabeled extension products under denaturing conditions. The same template is used, except the 40 bp primer has been phosphorylated at the 5' end with [γ-^{32}P]ATP (>5000Ci/mmole) and T4 polynucleotide kinase. The labeled oligo was purified using a NucTrap probe purification column (Stratagene) and then annealed with single-stranded M13 at equimolar concentrations (100nM). The reaction cocktail comprised:

9.5µg/ml single-stranded M13mp18 (+) strand DNA (Pharmacia)

52 ng/ml 40-mer

100µM each dNTP

1x cloned Pfu DNA polymerase buffer

and water to 50µl.

Single-stranded M13 DNA was mixed with primer, buffer and water. The mix was heated to 95°C for 2 minutes and then cooled to room temperature. The

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rest of the reaction components were added. Each reaction contained diluted cloned Pfu DNA polymerase, and varying amounts of PCNA, and RFC. Reactions were incubated at 72°C for varying times ranging from 1-30 minutes. The reactions were terminated by adding 3.3µl of stop dye (95% formamide/20mM EDTA/ 0.05% bromophenol blue/0.05% xylene cyanol). The reaction mixtures were then subject to polyacrylamide gel electrophoresis using 6-8% denaturing gels, and the gels are dried down and exposed to autoradiographic film. The size of the full length extension product was determined by carrying out primer extension using excess cloned Pfu DNA polymerase for 30 minutes.

B. Stimulation of nucleotide incorporation

The accessory factors were also tested for the capability of increasing dNTP incorporation by Pfu DNA polymerase or *P. furiosus* pol II DNA polymerase. This assay involves measuring dNTP incorporation into primed M13 DNA, by isolating and counting high-molecular-weight DNA bound to DE81 filter paper. A reaction cocktail is prepared as follows:

4 µg/ml single-stranded M13mp18 (+) strand DNA (Pharmacia)

219 ng/ml 40-mer (see Section 5(A) above)

1X cloned Pfu DNA polymerase buffer (Stratagene)

300 µM each dGTP, dATP, dCTP

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30 μM dTTP

5 μM ^{3}H-TTP (NEN NEG-221H)

To 10μl of reaction cocktail was added either 0.025 units cloned Pfu Polymerase (Stratagene) or 0.05 units *P. furiosus* pol II. *P. furiosus* pol II was PCR amplified using the DNA sequences described in reference 15 below, and recombinant CBP-DP1/DP2 DNA polymerase was cloned, expressed, and purified as described using the procedures outlined above in Section 1. To assay the temperature-dependence of PCNA enhancement (data in Figure 42), reactions were carried out for 10 minutes in the absence or presence of 100ng PCNA, using incubation temperatures ranging from 66-99°C.

The extension reactions were quenched on ice, and then 5μl aliquots were spotted immediately onto DE81 filters (Whatman). Unincorporated [^{3}H]TTP was removed by 6 washes with 2xSCC (0.3M NaCl, 30mM sodium citrate, pH 7.0), followed by one wash with 100% ethanol. Incorporated radioactivity was measured by scintillation counting.

This assay can be modified to allow improved detection of PCNA by reducing dNTP incorporation to background levels through the addition of 200 mM KCL to the reaction mix. PCNA alone or in combination with other accessory factors can be detected by restoration of Pfu's DNA polymerase activity.

The assay cocktail contains:

10 μg/ml single-stranded M13 mp 18 (+) strand DNA (Pharmacia

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cat# 27-1546-01)

100 ng/ml	40-mer primer (GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GTG C)
1X	cPfu buffer
200 mM	KCl
30 μM each	dATP, dCTP, dGTP
3 μM	dTTP
5 μM	^{3}H-dTTP (NEN cat# NET-221H) (100 μCi/mL)
100 U/ml	cloned Pfu polymerase

Recombinant accessory factors or fractions derived from native *P. furiosus* are assayed for their ability to restore polymerase activity to the above cocktail. 1 μl samples were added to 10 μl of reaction cocktail, and reactions were incubated at 72ºC for 30 minutes. Reactions were spotted onto DE81 filter papers, which were then washed and counted as described above.

C. ATPase assay.

One μl of RFC or helicase preparations were incubated with 1 μl of 4.5 μM ATP and 1 μCi of gamma labeled ^{33}P-ATP in 10mM Tris HCl (pH 8.3), 3.5 mM $MgCl_2$, and 75 mM KCl. The samples were incubated at 72ºC for 20 minutes before being spotted on PEI cellulose F (EM Science). After drying, the PEI cellulose was placed in a shallow reservoir of 0.4 M NaH_2PO_4 pH 3.5. The liquid front was allowed to migrate 4-5 cm before being removed from the liquid and dried. The samples were exposed to X-ray film for one hour. Evidence of ATPase activity in samples was obtained by looking for radioactivity migrating with the liquid front. The positive control (porcine ATPase) converts ^{33}P-γ-dATP

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to dADP + ^{33}P-γ P; the latter product migrates with the liquid front under these TLC conditions, while the ^{33}P-γ-dATP substrate remains near the origin. In some cases, product was quantified by excising the product spots from the PEI plate and then counting in a scintillation counter.

D. Gel shift assay.

A 38 base oligo (5' GGT TTT CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA GT 3') was incubated with RFA samples at 95ºC for 10 minutes, followed by 72ºC for 2 minutes, prior to loading on a 4-20% acrylamide gradient gel (Novex) in 1x TBE buffer. Bands were visualized by SYBER green staining (Molecular Probes) and UV illumination. DNA binding activity is monitored by looking for a retardation in the migration of the oligo (higher band) in the presence of RFA. Single-strand DNA binding activity is verified by showing a shift in band position using a single-stranded oligo but no shift using a double-stranded DNA duplex.

E. Helicase assay.

Radioactively labeled oligonucleotides with a 3' overhang or a 5' overhang were annealed to M13mp18 DNA (Pharmacia). The reactions were incubated with 0.5 µl of putative *P. furiosus* helicases in 50 mM Tris HCl, pH 8.5, 25mM KCl, and 5 mM ATP for 30 minutes at 55ºC. The positive control was generated

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by thermally melting the annealed oligo prior to loading. The samples were run on 4-20% gradient acrylamide gels in 1x TBE. The gels were dried and exposed to X-ray film. Samples with helicase activity will displace the annealed radiolabeled oligo from single-stranded M13 DNA. On a gel, helicase-displaced oligo will migrate with the same mobility as oligos melted off M13 DNA with heat (free oligo). In samples lacking helicase activity, oligo will still be bound to M13 and will migrate at a different position which will be identical to "template only" controls.

6. PCR Reactions.

PCR reactions were carried out under standard conditions. In general, amplification reactions (50 µl) contained 200-450 µM each dNTP, 1x PCR buffer, 50-200 ng of human genomic DNA template (or 100 ng Stratagene's Big Blue transgenic mouse genomic DNA for the 0.5 kb target), 100 ng of each primer, and 2.5-5U of TaqPlus® Long DNA polymerase blend, PfuTurbo DNA polymerase, or Taq2000 DNA (Stratagene) polymerase. TaqPlus® Long PCRs were carried out in 1x buffer including: 50 mM Tricine pH 9.0, 8 mM ammonium sulfate, 0.1% Tween-20, 2.3 mM $MgCl_2$, and 75 ng/_l BSA. PCRs using PfuTurbo or Taq2000 DNA polymerase were carried out with the PCR buffers provided with the enzymes (Stratagene). Reactions were cycled in 200 µl thin-walled tubes using any of the following temperature cyclers: Stratagene RoboCycler® 96 temperature cycler fitted with a hot top assembly, Perkin Elmer

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GeneAmp PCR System 9600, or MJ Research PTC-200 Peltier thermocycler.

The sequences of the PCR primers are given below:

23kb β-globin
Forward primer: 5'-CAC.AAG.GGC.TAC.TGG.TTG.CCG.ATT-3'
Reverse primer: 5'-AGC.TTC.CCA.ACG.TGA.TCG.CCT.TTC.TCC.CAT-3'

30kb β-globin
Forward primer: 5'-CTC.AGA.TAT.GGC.CAA.AGA.TCT.ATA.CAC.ACC-3'
Reverse primer: 5'-AGC.TTC.CCA.ACG.TGA.TCG.CCT.TTC.TCC.CAT-3'

2.1 kb Alpha 1 Anti-Trypsin
Forward primer: 5'-GAG.GAG.AGC.AGG.AAA.GGT.GGA.AC-3'
Reverse primer: 5'-GAA.AAT.AGG.AGC.TCA.GCT.GCA.G-3'

5.2 kb Alpha 1 Anti-Trypsin
Forward primer: 5'-GAG.GAG.AGC.AGG.AAA.GGT.GGA.AC-3'
Reverse primer: 5'-GCT.GGG.AGA.AGA.CTT.CAC.TGG-3'

0.5kb λ/lacI (transgenic mouse genomic DNA)
lambda primer: 5' GAC.AGT.CAC.TCC.GGC.CCG-3'
lacZ primer: 5' CGA.CGA.CTC.GTG.GAG.CCC-3'

The following temperature cycling conditions were used for the 23 and 30kb β-globin targets: 92_ for 2 min. (1 cycle); 92_ for 10 sec., 65_ for 30 sec., 68_ for 25 min. (10 cycles); 92_ for 10 sec., 65_ for 30 sec., 68_ for 25 min. (with a increase of 10 sec. added progressively to the extension time with each cycle)(20 cycles). The 2.1 and 5.2 kb targets were amplified as follows: 95_ for 1 min. (1 cycle); 95_ for 1 min., 58_ for 1 min., 72_ for 2 min. (for 2 kb target) or 5 min. (for the 5.2 kb target) (30 cycles); 72_ for 10 min. (1 cycle). The 0.5 kb

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target was amplified as follows: 94_ for 1 min. (1 cycle); 94_ for 1 min., 54_ for 2 min., 72_ for 1.5 min. (30 cycles); 72_ for 10 min. (1 cycle).

Results

1. PCNA.

P. furiosus PCNA was first identified in column fractions produced during fractionating native *P. furiosus* extracts. PCNA was co-purified with Pfu DNA polymerase during the Q and SP column procedures discussed above. Peak PCNA activity could be resolved from peak DNA polymerase activity using the heparin sepharose column, but all PCNA-containing fractions were contaminated with DNA polymerase activity. To isolate native PCNA, fractions that could restore DNA polymerase activity to salt-inactivated Pfu DNA polymerase were studied. Such "restoration" activity was detected in column fractions eluting off the Heparin sepharose (Figure 1). An active column fraction was then subject to SDS-PAGE and gel slices were excised and extracted to remove proteins. DNA polymerase activity was found in a gel slice recovered from a position in the gel corresponding to the migration of proteins between 64-98 kDa. In contrast, PCNA activity was recovered from a gel slice that was located at a position lying between the 30 and 36 kDa protein markers (Figure 2). A protein band, migrating at 35 kDa, was visible on SDS-PAGE gels. This protein was transferred to a PVDF membrane (Bio Rad) and sent for amino terminal sequencing. The N-terminal sequence of the 35 kDa protein was:

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PFEIVFEGAKEFAQLIDTASKL(H,I)DEAAFKVTEDG--MR (where (H,I) means either amino acid could be present, and - means that any amino acid could be present). A BLAST search of DNA sequence databases identified the 35 kDa protein as exhibiting significant homology to known eukaryotic PCNA sequences.

The sequence encoding *P. furiosus* PCNA was cloned in the pCALnEK vector using the PCNA PCR primers described above. The LIC-primers were designed using the DNA sequence for PCNA identified in the *Pyrococcus horekoshi* genome sequence database. Although closely related to archaea, *Pyrococcus horekoshi* is a different species of *Pyrococcus* than *Pyrococcus furiosus*. The translated N-terminus of the putative *Pyrococcus horekoshi* PCNA matches the chemically determined N-terminal sequence of native *Pyrococcus furiosus* PCNA. The DNA sequence of the pCALnEK clone encoding *Pyrococcus furiosus* PCNA is shown in Figure 3, and its translated amino acid sequence is shown in Figure 4. The predicted molecular weight of *P. furiosus* PCNA is 28 kDa although the apparent molecular weights of EK-digested recombinant PCNA and native PCNA are 38 and 35 kDa, respectively.

In addition to stimulating nucleotide incorporation by salt-inactivated Pfu DNA polymerase, both native and recombinant *Pyrococcus furiosus* PCNA preparations were shown to significantly increase the processivity of Pfu. When PCNA is added to primer extension reactions where a 5' radiolabelled primer is annealed to single stranded M13, the majority of the products generated at early time points are full-length and fewer short truncated products accumulated (Figure 5). These results indicate that PCNA has significantly increased the

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processivity of Pfu polymerase (number of bases added per polymerase/DNA binding event), and the overall rate of incorporation (nucleotides incorporated per unit time) is increased.

The effects of PCNA on *P. furiosus* pol II DNA polymerase were also tested. PCNA was shown to stimulate dNTP incorporation by both Pfu (pol I) and *P. furiosus* pol II DNA polymerases. Interestingly, the addition of PCNA altered the optimal reaction temperature for both DNA polymerases. Because DNA duplexes are unstable at elevated temperatures (>Tm), assaying DNA polymerases at temperatures approaching the optimal growth temperatures of hyperthermophilic archaea (>100°C) has been difficult. For the M13/40-mer duplex shown here, reaction temperatures above 75°C produce template instability, consistent with the drop in activity for both polymerases between 72 and 80°C. However, in the presence of PCNA, the primer/M13 duplex appears to be stabilized at temperatures >72°C, leading to even higher primer extension activity by both Pfu (pol I) and P. furiosus pol II DNA polymerases. Thus, the M13/oligo duplex remains annealed at temperatures greater than about 80°C.

These data indicate that the addition of PCNA can have other benefits besides enhancing the polymerization rate and processivity of Pfu (pol I) and P. furiosus pol II DNA polymerases. The use of PCNA should allow the use of these hyperthermophilic enzymes at higher temperatures than has been achieved to date. PCR amplification, DNA sequencing, and isothermal amplification reactions employ extension temperatures of $\leq$72°C to ensure

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stability of the primer/template duplex. However, this temperature is well below the expected temperature optimum of DNA polymerases from hyperthermophilic archaea like *P. furiosus*. It may be possible to use elevated extension temperatures during these polymerization reactions (e.g., >80°C), which would have the benefits of increasing polymerase activity (by operating closer to optimum reaction temperature) and reducing interference from secondary structure in DNA templates.

In addition, the apparent stabilization of primer/M13 DNA duplexes by PCNA may have utility in improving applications that require high stability of nucleic acid duplexes. For example, PCNA may enhance the specificity of probe hybridization reactions by allowing the use of more stringent annealing temperatures or reaction conditions (lower ionic strength).

The effect of adding PCNA without other accessory factors to PCR amplification reactions has been tested. In the absence of other accessory factors, relatively high concentrations of PCNA (100 ng-1 ug) can inhibit product synthesis by Pfu DNA polymerase. Lower concentrations of PCNA are tolerated in PCR amplification reactions (<100 ng). PCNA is functional and beneficial to PCR amplification reactions (Figures 6 and 7). PCNA can dramatically increase the yield of products amplified with DNA polymerase blends including *Taq*, *Pfu*, and *P. furiosus* dUTPase (PEF). In the blends that have been tested, Taq is present at 2.5-5U and Pfu is present at 0.156-0.3125 U. The dUTPase may be in the form of native PEF or recombinant dUTPase (P45) (See WO 98/42860) and present at 1-10 ng per reaction. PCNA enhances the processivity of the

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minor proofreading component in the DNA polymerase blend, while dUTPase is preventing dUTP incorporation (and subsequent Pfu inhibition), so that greater Pfu polymerase activity can be realized. The dUTPase activity is discussed in International Patent Application WO98/42860, which is incorporated in its entirety by reference. Therefore, PCNA in its optimal concentration should enhance archaeal DNA polymerases (such as Pfu, pol II), either alone, or in combination or blended with other non-proofreading DNA polymerases of eubacterial or archaeal origin. In addition, PCNA activity can be improved by the further addition of other accessory factors including *P. furiosus* RFC, RFA and helicase.

2. RFC.

Before this invention, the inventors were not aware of the availability of an archaeal genome sequence other than the sequence of *Methanococcus jannaschii.* Genome sequence of *Methanococcus jannaschii* contained ORFs, which exhibited significant DNA sequence homology to DNA replication proteins from eukaryotes, including one Family B DNA polymerase, two RFC subunits, and PCNA (Bult et al., 1996 (Reference No. 6)). In eukaryotes, the RFC complex is composed of five distinct subunits (one large subunit and 4 small subunits that are associated with ATPase activity) and is stimulated by PCNA. However, only two genes were identified in *Methanococcus jannaschii* as exhibiting homology to RFC subunits: one sequence was identified as a putative homolog of the large RFC subunit and a second sequence was identified as a

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putative homolog of one of the small subunits. Initially, PCR primers were based upon the DNA sequences of the putative *Methanococcus jannaschii rfc* genes. However, these primers did not amplify a PCR product from *P. furiosus* genomic DNA, presumably because of the divergence in DNA sequence between *Methanococcus jannaschii* and *Pyrococcus furiosus.*

The inventors used an alternative approach to clone *P. furiosus* RFC subunits. Amino acid sequence alignments between *Methanococcus jannaschii* and human RFC identified a 67-amino acid region with 52% identity. A portion of RFC was likely to be highly conserved among archaea, since it was relatively conserved between more distantly related organisms, i.e., humans and archaea.

A 200 bp sequence encompassing the region encoding the 67-amino acid region was amplified from *Methanococcus jannaschii* genomic DNA using the following primers: 5' GAT GAA AGA GGG ATA GAT and 5' ATC TCC AGT TAG ACA GCT. The *Methanococcus jannaschii* sequence was used to probe a *P. furiosus* genomic DNA library.

One positive genomic clone was recovered which contained the sequences encoding both the large and small subunits in tandem. The DNA sequence of the genomic clone is shown is Figure 8 and the translated amino acid sequence is shown in Figure 9. The genomic sequences of P38 and P55 are bracketed. The nucleotide sequence of P38 is nucleotides 197 to 2835 (the intein sequence is nucleotide 374 to 2028). The nucleotide sequence of P55 is nucleotides 2839 to 4281. Examination of the DNA sequence encoding the small *P. furiosus* RFC subunit (P98) revealed the presence of an intein. An

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intein had also been identified in the gene encoding the putative *Methanococcus jannaschii* small RFC subunit (Bult *et al*, 1996).

Expression constructs were prepared by subcloning the sequences encoding the large and small subunits into the pCALnEK vector. To facilitate expression, the intein was removed from the small RFC subunit clone by amplification with primers designed to anneal to the 5' and 3' regions flanking the intein sequence and to prime in a direction opposite to the intein. The amino acid sequences of the large RFC subunit and the small "intein-less" RFC subunit are shown in Figures 10 and 11.

Antibodies were raised in rabbits against the P55 and P38 subunits. The native RFC complex was purified from *P. furiosus* extracts by immunoaffinity chromatography using either immobilized anti-P55 or anti-P38 IgG. Western blot analysis of immunoaffinity-purified RFC complex shows the presence of both subunits regardless of the capture antibody (Figure 12), indicating that P38 and P55 form a complex in *P. furiosus* as do the large and small RFC subunits in eukaryotes. The protein composition of one native RFC preparation is shown in Figure 13. In addition to P55 and P38, there are other protein bands present which have not yet been identified.

The ATPase activity of the RFC preparations was tested (Figure 14). RFC subunits are ATPases. That is, they convert ATP to ADP and phosphate. RFC complex in eukaryotes is known to load PCNA clamp onto DNA in a process that typically requires the conversion of ATP to ADP and phosphate. Recombinant P55 and P38 exhibited ATPase activity when assayed separately. A mixture of

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P55 and P38 subunits was also found to exhibit ATPase activity that increased in the presence of PCNA, but not in the presence of primed M13 DNA. In contrast, native RFC purified by immunoaffinity chromatography exhibited ATPase activity which was further stimulated by both PCNA and DNA. As the eukaryotic RFC complex is stimulated by both PCNA and DNA, it appears that the native RFC preparation is fully functional, while the mixture of recombinant P55 and P38 may be only partially active. This conclusion was supported by primer extension studies in which a native RFC preparation from *P. furiosus* (Immunopurified) was shown to enhance the yield of full-length products synthesized with Pfu DNA polymerase in the presence of PCNA (Figure 15). In contrast, a mixture of recombinant P55 and P38 with similar ATPase activity showed less enhancement of primer extension by Pfu + PCNA. It is presently unknown whether the difference in activity between native and recombinant RFC is due to differences in the P55:P38 ratios or protein modification, or to the absence of additional proteins present in the native RFC preparations. One skilled in the art could determine a solution by attempting different ratios of P55 to P38 or different reaction conditions, or by adding additional protein factors such as the ones present in a native RFC preparation.

3. RFA.

The large subunit of eukaryotic RFA was used to search the archaeal protein databases with PSI-BLAST. Hits to archaeal sequences were examined.

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The inventors aligned corresponding sequences to identify the putative start and stop codons of the *RFA* sequence in the incomplete *P. furiosus* genome sequence. *P. furiosus rfa* sequence was PCR amplified and cloned into the pCALnEK vector. The DNA sequence and translated protein sequences are shown in Figures 16 and 17. The apparent molecular weight of the expressed fully denatured protein was consistent with the size expected from the translated DNA sequence (41 kDa).

To assess function, *P. furiosus* RFA was tested for single-stranded DNA binding activity in a gel shift assay (Figure 18). When RFA was incubated with a 38 base oligonucleotide, the migration of a percentage of the DNA was reduced, indicating that RFA does exhibit single-stranded DNA binding activity. In comparison, *E. coli* SSB was found to completely retard the oligo. The weaker single stranded DNA binding activity exhibited by *P. furiosus* RFA may be explained by use of insufficient protein, the presence of the CBP tag, or the use of suboptimal reaction conditions. The degree of migration of the oligo is related to the mass of the protein-DNA complex and the formation of protein multimers. *E. coli* SSB is known to form tetramers, but it is presently unknown whether *P. furiosus* RFA produces multimers as well.

The addition of *P. furiosus* RFA to amplifications carried out with Pfu and Taq DNA polymerases was shown to increase amplification specificity (Figures 19 and 21) and PCR product yield (Figures 20 and 21). The conditions were as described in Section 6 above. In Figure 21, *P. furiosus* RFA produced effects which were similar to those generated by *E. coli* single-stranded DNA binding

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protein (SSB; Stratagene's Perfect Match), including increased yield and amplification specificity and retardation of DNA migration at excess concentrations (5 μl). No evaluation of the relative performances of *P. furiosus* RFA and *E. coli* single-stranded DNA binding protein in PCR has been made; however, the increased thermostability of RFA should provide an additional benefit in temperature cycling.

4. Helicase.

Cells contain multiple helicases with specialized roles in a number of processes including replication, DNA repair, recombination, transcription, and translation. Known helicases have been classified into five families based upon sequence homology. Mechanistically, there are 2 classes of helicases depending upon whether unwinding requires a 3 ' overhang (3'-5' polarity) or a 5' overhang (5'-3' polarity), which is characteristic of helicases functioning in DNA replication. Archaeal replicative helicases were identified by identifying as many ORFs as possible in archaeal genomes that exhibited homology to any known eukaryotic helicase, regardless of specific metabolic role. No putative helicase sequences were excluded because helicase function between archaea and eukaryotes may be different. Moreover, the eukaryotic replicative helicase has not been conclusively identified. Using eukaryotic helicases, a PSI-BLAST search in the archaeal protein databases was conducted.

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Eight putative helicases meeting the criteria were selected for analysis. The incomplete *P. furiosus* genome sequence was examined to identify the putative start and stop codons of these sequences and to design PCR primers for cloning. The DNA sequences are shown in Figures 22-28, and 40, respectively; the translated protein sequences are shown in Figures 29-35, and 41, respectively. The apparent molecular weights of the expressed proteins were consistent with the sizes expected from the translated DNA sequence (see figure description of Figures 29-35). Future corrections in the incomplete *P. furiosus* genome sequence may define alternative start and stop sites.

Helicases act to displace the complimentary strand of DNA or RNA to uncover template for DNA polymerases, RNA polymerases, accessory factors, and repair factors. Helicases melt the complimentary strand in a process coupled to hydrolysis of ribo- or deoxyribonucleotides. Most helicases displace either a 5' overhang or a 3' overhang, but some helicases displace both templates or utilize different templates under different reaction conditions. Typically, a helicase will utilize one or more nucleotide triphosphates preferentially. To assess the function of the identified eight helicases, recombinant helicases were tested for ATPase activity. The ribonucleotide ATP was used, although other ribo- or deoxyribonucleotides may serve as the preferred substrate. The resulting recombinant proteins were incubated with ATP, and phosphate was detected after separation by TLC. The results in Figures 36 and 37 demonstrate that all eight recombinant helicases exhibit ATPase activity.

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Eight recombinant helicases were tested for helicase activity. The templates used included labeled oligonucleotides annealed to single-stranded M13mp18 DNA. The oligos had either 5' or 3' non-complementary ends. As shown in Figure 38, helicase 2 was able to displace oligos from both templates. This helicase also melted a template which had non-complementary 5' and 3' ends (data not shown). Such a forked template mimics the "bubble" formed by the replication fork. In addition, helicase 7 displaced the oligo with a free 3' end (Figure 38). The lack of detectable oligo displacement does not necessarily mean that the rest of the enzymes are not helicases, because lack of helicase activity may be attributed to the use of suboptimal buffers or reaction conditions, the presence of the N-terminal CBP tag, or the use of insufficient amounts of recombinant protein. Preliminary experiments showed that the addition of diluted preparations of helicase 2 or helicase dna2 to PCRs in combination with Pfu DNA polymerase can lead to increased PCR product yield (data not shown).

All documents mentioned in this application, including but not limited to, articles, books, reviews, patents and patent applications, are hereby incorporated by reference in their entirety into this specification.

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